Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: van Bon L, Affandi AJ, Broen J, et al. Proteome-wide analysis and CXCL4 as a biomarker in systemic sclerosis. N Engl J Med 2013;370:433-43. DOI: 10.1056/NEJMoa1114576

Supplementary Appendix

Proteome-wide Analysis and CXCL4 as a Biomarker in Systemic Sclerosis

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- Decision to Publish the Paper. L. van Bon, A.J. Affandi, W. van Heerde, B. Homey, M. Roest, M. Trojanowska, R. Lafyatis, T.R.D.J. Radstake.

There were no agreements concerning confidentiality either with sponsors or institutions.
No subgroup analysis was performed.

Materials and Methods

Patient and Phenotypes

The presence of systemic sclerosis specific autoantibodies, anti-topoisomerase I and anti-centromere, was assessed by passive immunodiffusion against calf thymus extract (Inova Diagnostics, San Diego, CA) and indirect immunofluorescence of HEp-2 cells (Antibodies Inc., Davis, CA), respectively. To investigate the correlation between the level of skin fibrosis and CXCL4, the modified Rodnan skin score (mRSS) was assessed[1] within 2 months before or after sampling of plasma. As we aimed to investigate systemic sclerosis patients that reflect the daily clinical practice, all use of medications was allowed. No differences were observed when patients with different therapeutic regimens were compared (data not shown). The presence of pulmonary fibrosis was determined by high-resolution computed tomography scan and further defined as a forced vital capacity (FVC) < 70% of the predicted value and a diffusion capacity of the lung for carbon monoxide (DLCO) of less than 70% of predicted. Pulmonary arterial hypertension (PAH) was diagnosed by right heart catheterization and considered confirmed when the hemodynamic definition of PAH; mPAP ≥25 mm Hg with a pulmonary capillary wedge pressure (PCWP) of ≤15 mm Hg and a pulmonary vascular resistance of >3 Wood units was met. The time-to-develop fibrosis or PAH was assessed in patients with high or low CXCL4 levels up to 24 months from the first determination of the molecule. A separate analysis was conducted in all the patients with PAH and in the subset of PAH patients with isolated PAH, that is in those without any detectable lung fibrosis before the diagnosis of PAH (n=10 in high CXCL4 group and n=9 in low CXCL4 group); when the two populations were separately analyzed no differences in the survival estimates and in the hazards between groups were observed. In these analyses, 10 patients were lost to follow up for high-resolution computed tomography (HRCT) assays (4 with low and 6 with high CXCL4) and 11 were lost to follow-up for assessment of PAH (5 with low and 6 with high CXCL4).

Liver biopsy and assessment of fibrosis

For the purpose of this study we assessed 93 patients with suspected liver disease caused by hepatitis infection or alcohol abuse and grouped them according to the severity of liver fibrosis. Liver biopsies for histological assessment of fibrosis were obtained using a 1.6 mm diameter

Menghini-type needle via right intercostal approach with local lidocaine anesthesia (Hepafix, Braun, Melsungen, Germany). The biopsy site was marked by ultrasound examination. No side effects occurred and good quality samples were obtained. Biopsy specimens were immersed in 2% formaldehyde and subsequently fixed with paraffin. A van Gieson stain for collagen was assessed for the presence of pericellular and perivenular fibrosis, as well as the expansion of the portal tracts. The specimens were staged according the METAVIR scoring system, where F0 indicates the lack of fibrosis, F1 corresponds to portal fibrosis without septa, F2 to the presence of few septa, F3 to the finding of numerous septa without cirrhosis. F4 indicates the presence of cirrhosis. Cirrhosis (F4) was diagnosed by histology, ultrasonography or on clinical grounds. We included a total of 93 patients, a total of 45 patients had a clinical diagnosis of cirrhosis, while 48 patients had only minimal or absent liver fibrosis. (F0 in 26 patients; F1 in 22 patients).

Cell isolation and purification

To minimize intra- and inter-assay differences, for the cell experiments described below blood was drawn simultaneously on at least 6 subjects, consisting of 1-2 healthy individuals, limited cutaneous systemic sclerosis, late diffuse cutaneous systemic sclerosis and early diffuse cutaneous systemic sclerosis patients. First peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll gradient centrifugation as previously described.[2] Thereafter, the isolation of plasmacytoid dendritic cells (pDCs) was performed using magnetic bead isolation (MACS) using the pDC (CD304/BDCA4) positive selection kit according to the manufacturer's protocol (Miltenyi Biotec, Auburn, CA). The positive fraction was reapplied to the separation column to enhance purity. Purity of the pDC population was investigated using flow cytometry for the markers BDCA2, CD19, BDCA-1 (CD1c), BDCA-3, CD14 and CD123 (all Miltenyi Biotec, Auburn, CA) (**Fig. S1A).** Intracellular staining of pDC for CXLC4 was performed after stimulation of plasmacytoid dendritic cells for four hours with PMA (50 ng/ml; Sigma-Aldrich, St. Louis, MO) and Ionomycin (1 µg/ml; Sigma-Aldrich, St. Louis, MO) in the presence of Golgiplug (1 µl/ml; BD Biosciences, San Jose, CA). Next, for intracellular detection of CXCL4, cells were washed, fixed and permeabilized according to the manufacturer's protocol (eBioscience, San Diego, CA) and incubated with phycoerythrin-conjugated anti-CXCL4 antibody (IC7952P, R&D systems, Minneapolis, MN). The purity of pDC was always > 96%. As an extra validation, the frequency of dendritic cell subsets (plasmacytoid dendritic cells, myeloid

DC type I (MDC1) and myeloid DC type I (MDC2)) in the circulation was simultaneously monitored using the blood dendritic cell enumeration kit (Miltenyi Biotec, Auburn, CA). To exclude the possibility that the positive selection of plasmacytoid dendritic cells would have influenced our results, in three consecutive experiments the blood samples were split and plasmacytoid dendritic cells were both positively selected as described above but also negatively selected using the Diamond pDC isolation kit (Miltenyi Biotec, Auburn, CA). In these experiments phenotype and cytokine production was noted to be similar between positively and negatively selected plasmacytoid dendritic cells (data not shown). After isolation of plasmacytoid dendritic cells, the cells were suspended in RPMI (10^6/ml) and incubated at 37°C for two hours. In some experiments, plasmacytoid dendritic cells were stimulated with toll-like receptor ligands for TLR7/8 (R848) or TLR9 (CpG or CpG control) for 24 h after which supernatants were collected and stored at -80°C. Anti-CXCL4 (ab9561, Abcam, Cambridge, UK), or Heparinase, a natural CXCL4 antagonist, was used to counteract the effect of CXCL4. As a control anti-CXCL12 (LS-B7489, LifeSpan Biosciences, Seattle, WA) was used as an isotype matched irrelevant antibody.

Human Dermal Microvascular Endothelial Cells and Human Umbilical Vein Endothelial Cells

Human dermal microvascular endothelial cells (HDMECs) were isolated from foreskin tissue as previously described.[3] These cells were cultured on collagen type I coated plates in EBM medium supplemented with 10% fetal bovine serum (FBS), EC growth supplement mix, at 37°C under 5% CO2 in air. The culture medium was changed every other day. HDMECs harvested between passage 2 and 4 were used for experiments. Tissue culture reagents, antibiotic-antimycotic solution (penicillin, streptomycin, and amphotericin B) was purchased from Life Technologies BRL (Grand Island, NY), EBM kit from Lonza (Walkersville, MD) and FBS from HyClone (Logan, UT). Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords from healthy donors after obtaining informed consent. Cells were cultured in RPMI-1640 Dutch modified (Gibco-Invitrogen, Carlsbad, CA) supplemented with heat-inactivated 10% FBS and 10% human serum at 37°C and 5% CO_2, in 0.2% w/v gelatinized (Sigma–Aldrich, St. Louis, MO) flasks or plates (Corning, Corning, NY). HUVECs grown in a 12-well plate were treated with CXCL4 (1 and 5 μg/mL, Haematologic Technologies Inc., Essex Junction, VT), after 24 h, cells and supernatants were collected for RNA or protein analysis. The

systemic sclerosis plasma was pooled from 5 patients with diffuse systemic sclerosis. Anti-CXCL4 (ab9561, Abcam, Cambridge, UK) was used to counteract the effect of CXCL4 in these studies. ET-1 immunoassay (Assay Designs, Ann Arbor, MI, cat no. 900-020A) was performed according to manufacturer's protocol. Cultured HDMECs were treated with CXCL4 (100ng/ml) and/or VEGF (100ng/ml, positive control) for 24 h. For cell proliferation assay, cells were seeded in a 96-well plate at a density of 20000 cells/well and were allowed to adhere overnight. At 48 h cell proliferation was determined by CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, 20 µl of One Solution Reagent were added to each well followed by 3 h of incubation at 37°C, and absorbance was read at 450 nm. Results were calculated as percent change in proliferation in comparison to control from five separate experiments.

Measurement of secreted proteins

Supernatants from Plasmacytoid Dendritic Cell in Culture

After two hours of incubation in RPMI cultured plasmacytoid dendritic cells (pDC) were centrifuged, supernatants stored at -80°C, cell purity tested by flow cytometry and RNA isolated from the remaining pDC. Type I IFN was assayed in pDC supernatants using an IFNα specific ELISA (Abnova, Heidelberg, Germany) and retested using the IFNα multiple subset ELISA (CellSciences, Canton, MA). Multiple cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFNγ, TNFα, IL-1β, IL-5, IL-7, IL-12, IL-13, IL-17F, G-CSF, MCP-1, MIP1β, IFNα, IL-23, IL-17A and IL-1α) were tested in pDC supernatants using the Bio-Plex system (Bio-Rad, Hercules, CA). The sensitivity of the cytokine assay was <5 pg/ml for all cytokines measured. β-thromboglobulin (β-TG) was measured using a β-TG specific ELISA (Calbiotech, Spring Valley, CA) and CXCL4 levels in supernatant were measured using a CXCL4 specific ELISA (R&D system, Minneapolis, MN) according to manufacturers' protocols.

Plasma Measurements

Levels of CXCL4, CCL5, CCL2, CCL18 and CXCL10 were determined in plasma using semi automated ELISA on a TECAN Freedom EVO 150 robotic pipetting station. Commercial antibody duosets: Dy795, Dy393, Dy278, Dy394 and Dy466 (R&D Systems, Abbington, UK) were used to quantify CXCL4, CCL2, CCL5, CCL18 and CXCL10, respectively. Since plasma

samples originated from different collaborating samples and to correct for differences in sample handling (e.g., platelet activation) in all plasma samples β-TG and vWF were measured simultaneously. No marked differences in these two markers were observed between systemic sclerosis patients and/or healthy individuals that would suggest different sample handling excluding the possibility that differences in CXCL4 were due to inter-cohort differences. Each antigen was measured on a separate Nunc MaxiSorp 384 well ELISA plate (Thermo Fisher Scientific, Roskilde, Denmark). For each ELISA procedure we coated plates for 2 h with optimized capture antibody concentration of 1.0, 1.0, 0.5, 0.5 and 1.0 µg/mL for CXCL4, CCL5, CCL18, CCL2 and CXCL10, respectively. Unbound antibodies were washed with five steps phosphate-buffered saline (PBS)/0.5%Tween. Plasma samples were diluted 1/80 for CXCL4, 1/10 for CCL5 and 1/4 for CCL18, CCL2 and CXCL10 and then added to the plate with the corresponding capture antibody. Each plate contained four calibration curves consisting of standard plasma sample with known CXCL4, CCL5, CCL18, CCL2 and CXCL10 concentrations. Dilutions were made in PBS/1% BSA and incubated for 2 h on the capture antibodies. Unbound antigens were removed with five wash steps with PBS/0.5% Tween. For CXCL4, CCL5, CCL18, CCL2 and CXCL10 we added optimized detection antibody concentration of 0.25, 0.05, 1.0, 0.2 and 0.25 µg/mL, respectively. After three washing steps with PBS/0.5% Tween, a concentration of 0.15 µg/mL streptavidin/horseradish peroxidase (HRP, M2051, Sanquin, Amsterdam, the Netherlands) was added for 2 h to bind the biotin on the detection antibody. After three wash steps with PBS/1% BSA, the amount bound antigen concentrations were quantified after addition of Supersignal West Pico Chemiluminescent Substrate (#34080, Thermoscientific, Rockford, IL) on a Spectramax Luminescence Microplate Reader (Molecular Devices, Sunnyvale, CA). Plasma levels of von Willebrand factor were detected, using the same semiautomatic procedure, using 0.78 µg/mL mouse monoclonal directed against human vWF (A0082, Dako, Glostrup, Denmark) as capture antibody and 0.28 µg/mL HRP-conjugated rabbit polyclonal against human vWF (P0226, Dako, Glostrup, Denmark) as detection antibody. To guarantee independent measurements, it was decided to do all laboratory measurements by investigators that were blinded for the disease state of the blood samples.

Proteomics

To study a wide range of (unknown) proteins present in the supernatant of pDC after two hours of incubation on RPMI 1640 medium without additions, surface-enhanced laser desorption/ ionization–time-of-flight mass spectrometry (SELDI-TOF-MS) was applied. The SELDI-TOF-MS technology (Ciphergen Biosystems, Fremont, CA) consists of the protein chip array, mass spectrometer and data analysis software. Optimal conditions for pDC supernatant profiling were determined by testing three protein chip array surfaces (Q10, CM10 and IMAC-Cu) with different binding/washing conditions and dilutions of supernatant. The binding/washing buffer for the anionic exchange arrays (Q10) contained 0.1% Triton X-100 with 0.1 M Tris–HCl (pH 8) or 0.1 M sodium acetate (pH 6) and that for the cationic exchange arrays (CM10) contained 0.1% Triton X-100 with 0.1 mM ammonium acetate (pH 4.5 or pH 6). Both Q10 and CM10 chips were pre-incubated with binding buffer. The metal affinity-binding IMAC-Cu chips were loaded with 50 µl 0.1 M copper sulfate by vigorous shaking for 10 min. After washing with water, the chip surface was neutralized using 150 µl sodium acetate buffer pH 4 followed by a short wash with water and pre-incubation with binding buffer (0.1 M Tris–HCl, pH 7.4) containing 0.1% Triton X-100 and 100 mM or 500 mM NaCl. pDC samples were diluted 1:10 or 1:100 in binding buffer (total volume of 100 µl) and applied at random and in duplicate to the chip and incubated for 1 h shaking on a mixer. Afterwards, spots were washed six times with 150 µl binding buffer for 10 min (three times with and three times without 0.1% Triton X-100). Before application of the matrix (Sinapinic acid (SPA); Ciphergen Biosystems) in 50% acetonitrile/0.1% trifluoroacetic acid, another short wash with HPLC-grade water was performed and the chips were air dried for 10 min. The matrix was applied twice (0.8 µl each time, 1 min apart) and the chips were air dried prior to reading on a ProteinChip Reader IIC instrument (Ciphergen Biosystems), using the following settings: detector sensitivity 9; detector voltage 2900; positions 20–80 were read with an increment of 10; 50 laser shots were collected on each position; two warming shots were fired at each position; lag time focus of 241 ns; laser intensity 180. Calibration was done with a 7–30 kDa proteins mixture. After baseline subtraction, peak labeling was performed with CiphergenExpress Software (version 3.0) for peaks with a signal-to-noise (S/N) ratio ≥3 in the *m*/*z* range from 1.5 to 30 kDa, and then normalized by total ion current.

Western Blot Analysis of ET-1 and FLI-1.

Cells were collected and washed with phosphate-buffered saline (PBS). Cell pellets were suspended in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, and 1 mM glycerophosphate with freshly added phosphatase inhibitors (5 mM sodium fluoride and 1 mM Na_3VO_4) and a protease inhibitor mixture (Sigma-Aldrich, St. Louis, MO). Protein concentration was quantified using the BCA Protein Assay kit (Pierce, Rockford, IL). Equal amounts of total proteins per sample were separated via SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in milk in Tris-buffered saline with Tween 20 (TBST) overnight at 4°C and probed with primary antibody overnight at 4°C. After Tris-buffered saline (TBS) washes, membranes were probed with HRP-conjugated secondary antibody against the appropriate species for 1–2 h at room temperature. Antibodies were used as following: goat anti human ET-1 (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:500 dilution, mouse anti-human FLI-1 (Invitrogen, Carlsbad, CA) at a 1:500 dilution, monoclonal mouse anti-human β-actin (Sigma-Aldrich, St. Louis, MO) at 1:5000 dilution. Protein levels were visualized using ECL reagents (Amersham Biosciences, Piscataway, NJ).

Immunohistochemistry on Human Skin Sections

Skin samples from nine patients with diffuse systemic sclerosis and six healthy controls were immediately frozen in liquid nitrogen and stored at -80°C. For immunofluorescence analysis of the expression of BDCA2 and CXCL4, skin sections were fixed with paraformaldehyde (4%). Subsequently, the skin sections were incubated with 1% H_2O_2 in methanol for 30 min. Blocking steps were performed using donkey serum (5%). Antibodies directed against human BDCA2 (goat polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) as well as CXCL4 (rabbit polyclonal; Abcam, Cambridge, UK), or corresponding isotype control antibodies were added overnight at 4°C and followed by washes. Afterwards, the skin sections were incubated with secondary antibodies (donkey anti-goat Alexa Fluor 555, donkey anti-rabbit Alexa Fluor 488, Invitrogen, Carlsbad, CA) for 45 min as well as DAPI (Sigma-Aldrich, St. Louis, MO) for 10 min. Sections were fixed with Fluoromount-G (SouthernBiotech, Birmingham, AL). Immunoreactions were detected using the Axiovert 200M microscope and visualized by the software Axiovision 4.7 (Zeiss, Oberkochen, Germany).

Subcutaneous CXCL4 Injection Model.

Animals

Wild type C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). All mice were maintained at the Boston University School of Medicine Laboratory Animal Sciences Center. Mouse experiments were conducted under a protocol approved by the Boston University, Institutional Animal Care and Use Committee and in accordance with AAALAC guidelines.

***In Vivo* Administration of CXCL4**

Recombinant murine CXCL4 (Peprotech, Rocky Hill, NJ) was administered subcutaneously to 6-8 week old C57BL/6 mice using two delivery methods. For the pump model, mini-osmotic pumps (Alzet, Cupertino, CA) containing 1 μg of CXCL4 in a total of 200 μl volume were subcutaneously implanted for 7 days as described previously.[4] Each pump was designed to deliver agent at the rate of 1 μl per hour over 7 days. For the daily subcutaneous injection model, 500 ng of CXCL4 in 100 μl PBS with 0.05% mouse albumin (Alpha Diagnostic, San Antonio, TX) was injected subcutaneously for 7 or 28 consecutive days. At the designated time point, mice were sacrificed and the skin (~1 cm^2) surrounding or distal to the pump outlet or injection site was homogenized in TRIzol (Invitrogen, Carlsbad, CA) for total RNA preparation, or fixed in formalin for histology.

Mouse Skin Histology and CD45 Immunohistochemistry

Mouse skin was fixed in acetic acid–zinc-formalin (Newcomersupply, Middleton, WI), embedded in paraffin, and sectioned at 4 μm. To visualize skin morphology, deparaffinized sections were stained with hematoxylin for 2 x 2 min and eosin for 2 min (Surgipath, Leica, Richmond, IL), and mounted. For immune cells identification, CD45 immunohistochemical staining was performed using the ABC Vectastain kit (Vector Laboratories, Burlingame, CA). In brief, deparaffinized sections were rehydrated, steamer-heated for 20 min in BD Retrievagen A (BD Biosciences, San Jose, CA), incubated with Fc receptor blocker, background buster (Innovex Biosciences, Richmond, CA), and ABC blocking serum (Vector Laboratories, Burlingame, CA), and stained with rat-anti mouse CD45 as primary antibody (BD Biosciences, San Jose, CA). The subsequent incubations with secondary antibody, ABC complex (Vector Laboratories,

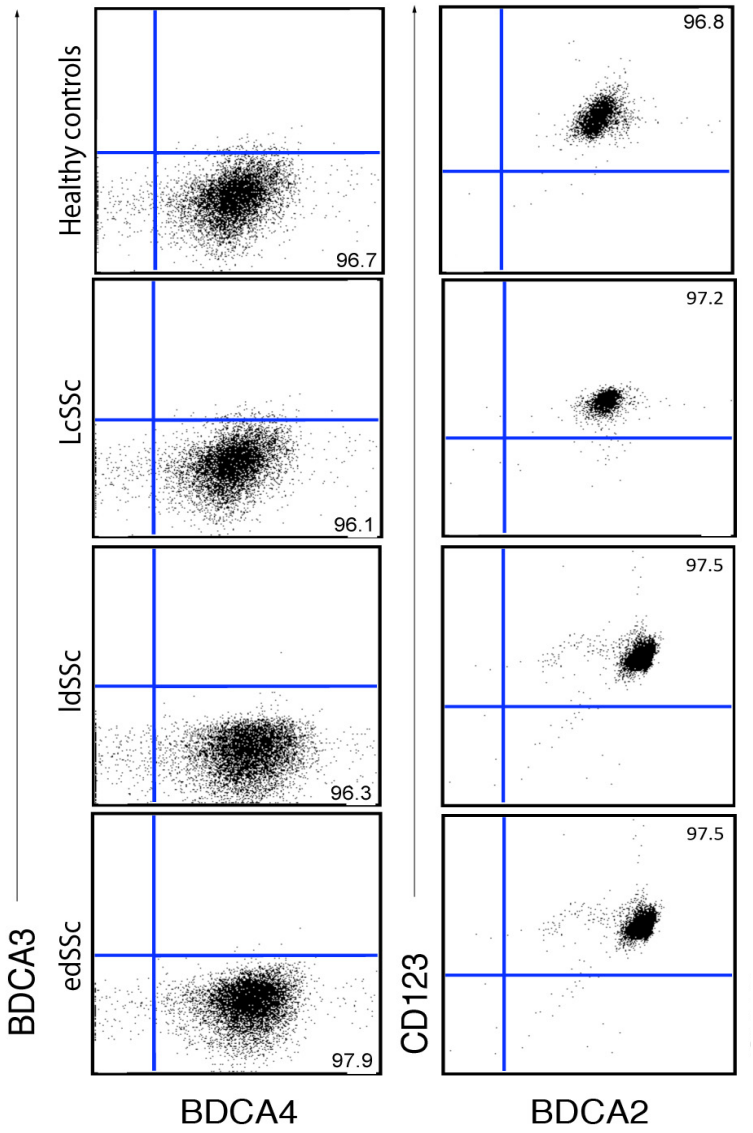
Burlingame, CA), and DAB as a substrate (Dako Corp, Carpinteria, CA) were then performed according to manufacturer's instructions. Images were acquired using a bright field microscope at 4x or 20x magnification (BH2 and BX41 Olympus, Center Valley, PA). Skin layer thickness and cell count were measured using ImageJ software (NIH, Bethesda, MA).

RNA Preparation and Quantitative PCR (qPCR)

Total RNA was extracted and purified from cells using RNeasy kit or from mouse skin using TRIzol (Invitrogen, Carlsbad, CA). cDNAs were developed from 200-300 ng of total RNA using Superscript II reverse transcriptase and random primers (Invitrogen Life Technologies, Rockville, MD). TaqMan primers for human CXCL4 (HS00236998_m1), human 18s rRNA (4314913E), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4352339E), mouse Ccl2 (Mm00441242_m1), mouse Tnf-α (Mm00443259_g1), mouse Ifit1 (Mm00515153_m1), mouse Mx2 (Mm00488995_m1), mouse Serpine1/PAI-1 (Mm00435858_m1), mouse Thbs1 (Mm01335418_m1), mouse Cd45 (Mm01293575_m1), mouse Icam-1 (Mm00516024_g1), mouse Edn1 (Mm00438656_m1) were purchased from ABI (Applied BioSystems Inc., Foster City, CA). SYBR green primer sets were mouse E-selectin 5'-tcctctggagagtggagtgc-3' (forward) 5'-ggtgggtcaaagcttcacat-3' (reverse), human Fli-1 5'-ggatggcaaggaactgtgtaa-3' (forward) 5'-ggttgtataggccagcag-3' (reverse), human Et-1 5'-gctcgtccctgatggataaa-3' (forward) 5'-ccatacggaacaacgtgct-3' (reverse), and human GAPDH 5'-ggtctcctctgacttcaaca-3' (forward) 5'-agccaaattcgttgtcatac-3' (reverse). Relative quantification of gene expression using TaqMan Master Mix or SYBR green chemistry was measured in ABI StepOnePlus system. Gene expression was normalized to housekeeping genes 18s ribosomal RNA, GAPDH, or β2-MG and relative expression of each gene was calculated using a ΔΔCt formula.[5]

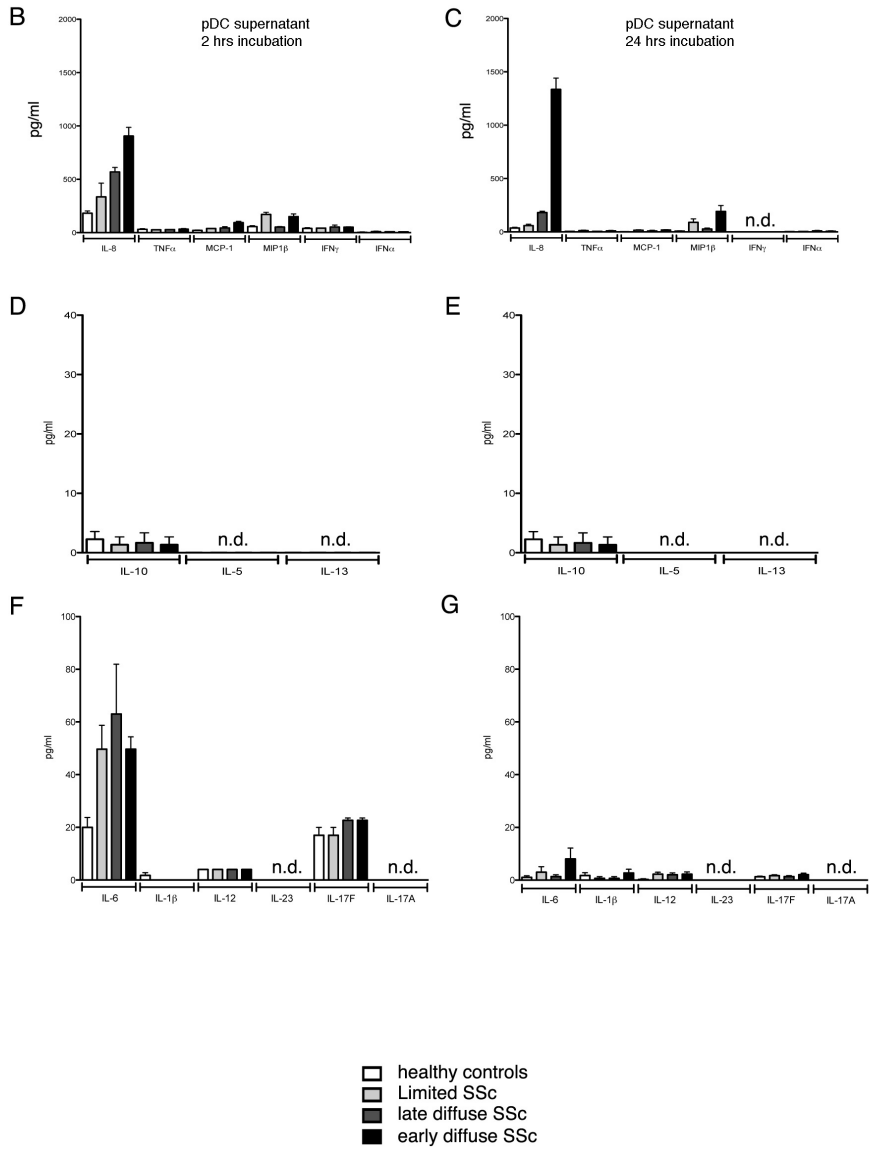
Supplementary Figures

Figure S1A. Flow Cytometry in Plasmacytoid Dendritic Cells in Systemic Sclerosis.



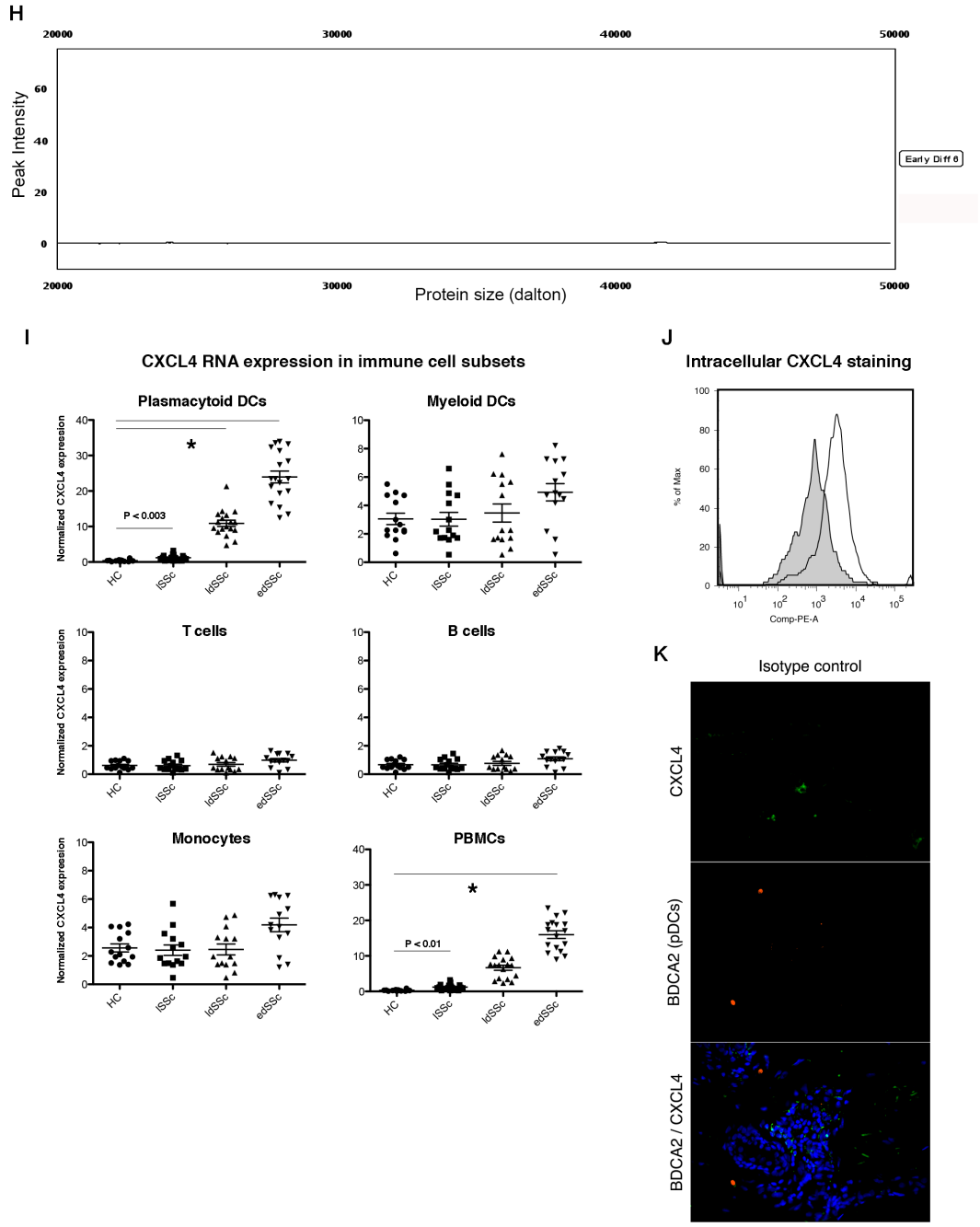
Panel A depicts plasmacytoid dendritic cells from healthy donors, patients with limited cutaneous systemic sclerosis (lcSSc), late diffuse cutaneous systemic sclerosis (ldSSc) and early diffuse cutaneous systemic sclerosis (edSSc) patients show similar expression of the markers BDCA4, BDCA2 and CD123. Expression of these markers was tested in 6 independent experiments comprising a total of 16 systemic sclerosis patients and 11 healthy controls. This panel depicts one representative experiment. Throughout all experiments purity was > 96%.

Figure S1B-G. Cytokine Production by Freshly Isolated Plasmacytoid Dendritic Cells from Healthy Controls and Systemic Sclerosis Patients.



Panel B and C represent the production of IL-8, TNFα, MCP-1, MIP1β, IFNγ, and IFNα by plasmacytoid dendritic cells from healthy individuals or systemic sclerosis patients after two and twenty-four hours of incubation, respectively. **Panel D and E** show the secretion of the Th2 markers IL-10, IL-5 and IL-13 whereas **Panel F and G** show the secretion of IL-6, IL-1β, IL-12, IL-23, IL-17F and IL-17A by plasmacytoid dendritic cells cultured from healthy controls or systemic sclerosis patients after the indicated time points.

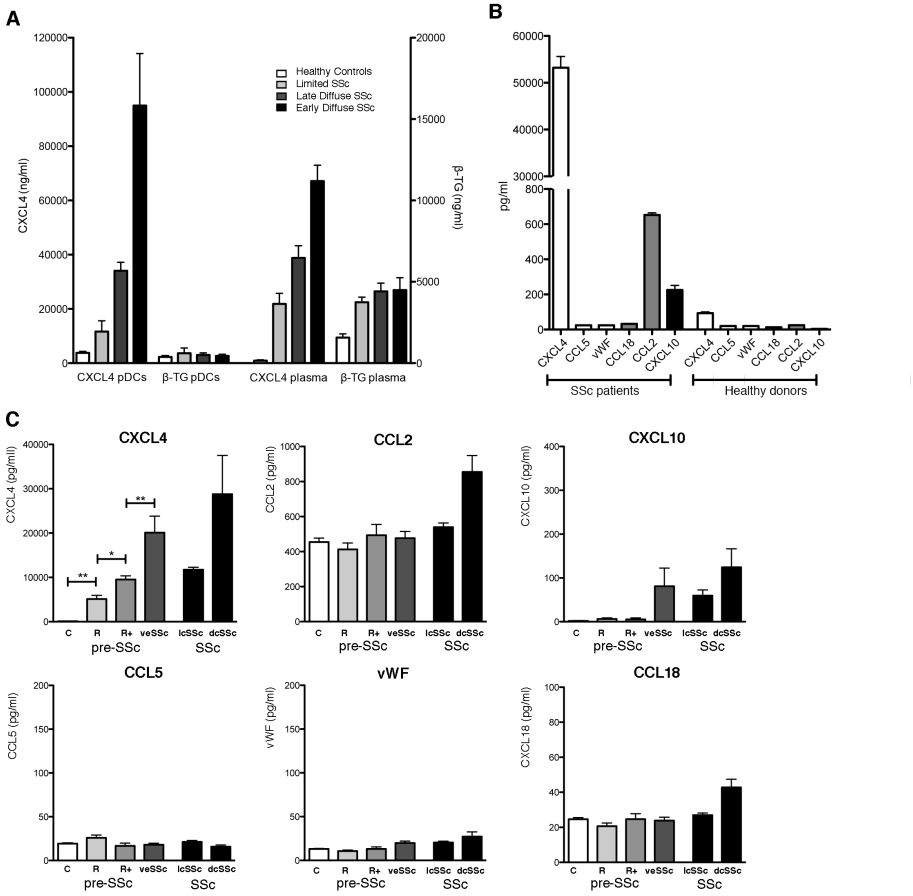
Figure S1H-K. Plasmacytoid Dendritic Cells from Systemic Sclerosis Patients Preferentially Secrete CXCL4.



Panel H displays the full proteome profile beyond 20kD from the supernatant of plasmacytoid dendritic cells from one representative systemic sclerosis patients with early diffuse disease. As in other experiments, no other peaks were present beyond 20kD. **Panel I** depicts CXCL4 RNA expression in various cell subsets isolated from healthy controls (n=12) and patients with limited

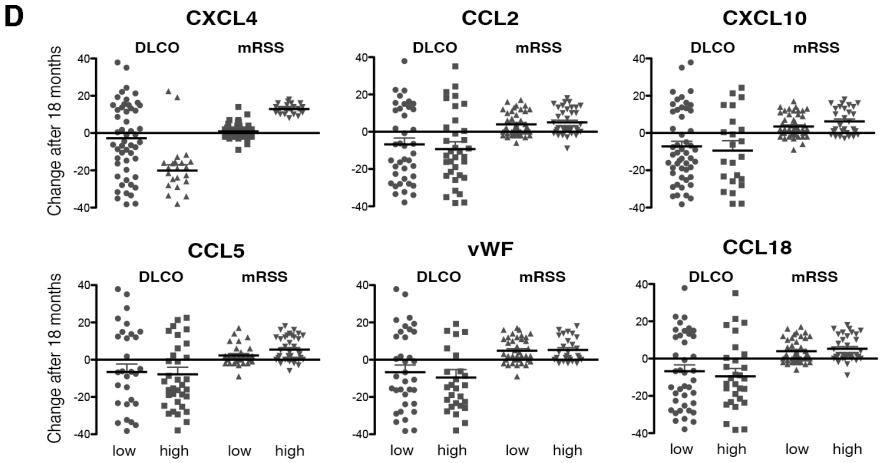
(lSSc, n=14), late diffuse (ldSSc, n=14), early diffuse systemic sclerosis (edSSc, n=14) from 7 independent experiments. Asterisk indicates P – values < 0.001. Intracellular expression of CXCL4 in plasmacytoid dendritic cells is shown in **Panel J**. **Panel K** represents separate and overlayed images of isotype controls for plasmacytoid dendritic cell marker BDCA2 (red), CXCL4 (green) and DAPI-labeled nuclei (blue), on a frozen skin section.

Figure S2A-C. CXCL4, CCL2 and CXCL10 Levels Are Increased in The Circulation of Systemic Sclerosis Patients and Only CXCL4 Correlates with Scleroderma Risk Unassociated with Platelet Activation.



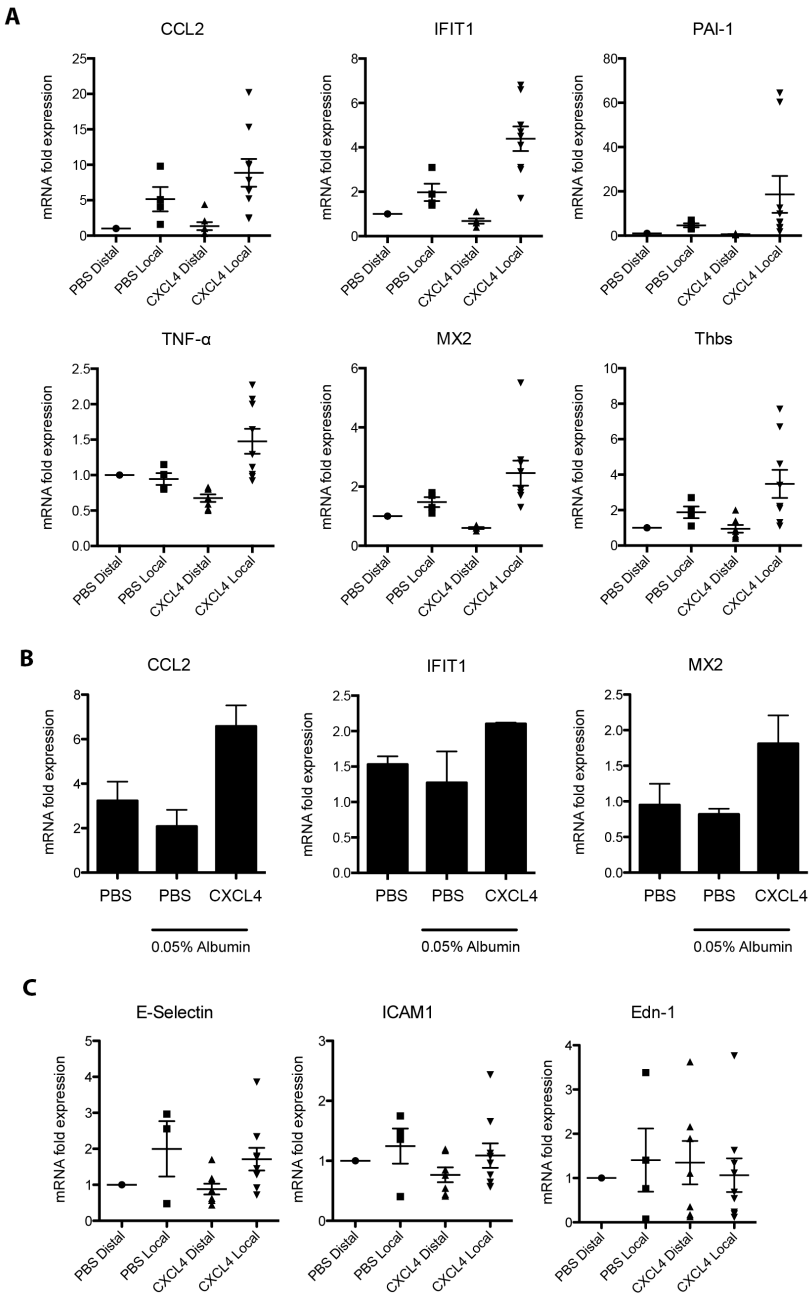
Panel A displays the absence of β-thromboglobulin (β-TG) in the plasmacytoid dendritic cells supernatant that excludes platelet contamination (n=119) and increased levels of β-TG in the circulation of systemic sclerosis patients compared to healthy controls. However, β-TG levels do not mirror CXCL4 plasma levels indicating that the CXCL4 in the circulation of systemic sclerosis patients is mainly coming from plasmacytoid dendritic cells. CXCL4 levels in systemic sclerosis patients (n=441) by far exceed those of chemokines previously suggested to be associated with systemic sclerosis phenotype (**Panel B**) and those observed in healthy donors (n=128). **Panel C** represents the circulating levels of CXCL4, CCL2, CXLC10, CCL5, vWF and CCL18 in scleroderma patients, healthy donors (C, n=128) and in comparison with individuals with primary Raynaud's phenomenon (R, n=16), persons with Raynaud's phenomenon and anti-nuclear antibodies (R+, n=10) and those with very early systemic sclerosis (veSSc, n=30). Asterisk indicates *P* – values < 0.001**.**

Figure S2D. CXCL4, CCL2 and CXCL10 Levels Are Increased in The Circulation of Systemic Sclerosis Patients and Only CXCL4 Correlates with Scleroderma Risk Unassociated with Platelet Activation.



Panel D shows that in a prospective cohort of 79 patients followed over 18 months, only a high baseline of CXCL4, but not, CCL2, CXLC10, CCL5, vWF and CCL18, predicted a worse outcome both of lung function (capacity of the lung for carbon monoxide (DLCO)) as well as for skin progression (modified Rodnan skin score (mRSS)).

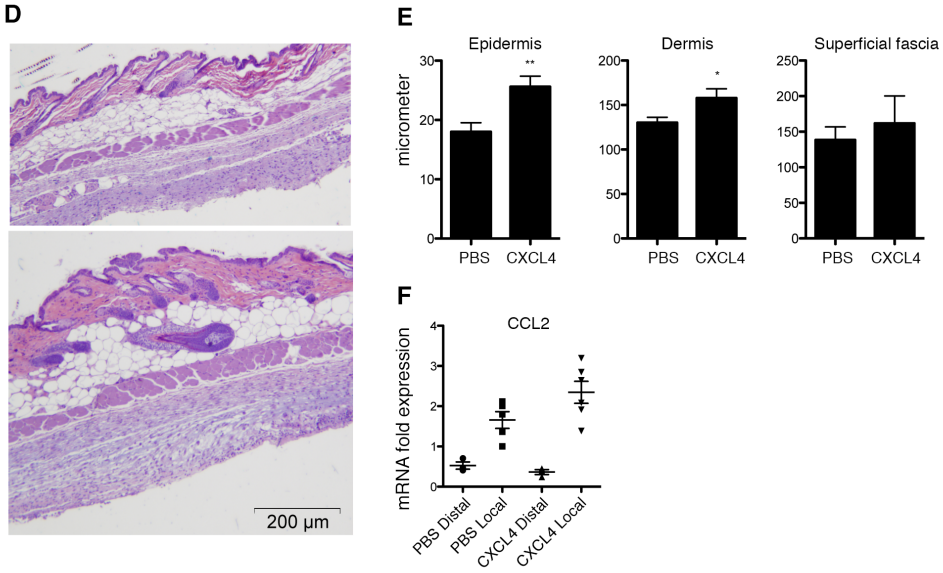
Figure S3A-C. CXCL4 Induces Systemic Sclerosis-like Effects in Subcutaneous Pump and in Subcutaneous Injection Models.



Panel A shows gene expression of wildtype-C57BL/6 mice treated with CXCL4 or phosphate-buffered saline (PBS) control for 7 days using the subcutaneous implanted pump model. Skin area located distally or locally to the pump outlet was isolated and mRNA expression for CCL2, TNF-α, IFIT1, MX2, PAI-1 and Thbs induced by CXCL4 were evaluated using qPCR as compared to the PBS controls (PBS local n=4, CXCL4 local n=7). **Panel B** displays that based on

mRNA expression of CCL2, IFIT1, and MX2, the presence of 0.05% mouse albumin in PBS as an additional unrelated control protein in a 7-day daily subcutaneous injection model did not lead to any noticeable differences (PBS n=3, PBS 0.05% albumin n=2, CXCL4 0.05% albumin n=3). **Panel C** shows that using qPCR, mRNA expression of the endothelial cell activation markers E-selectin, ICAM1 and Endothelin-1 (Edn-1) after 7 day treatment of CXCL4 in a pump model were comparable to the PBS controls (PBS local n=4, CXCL4 local n=7).

Figure S3D-F. CXCL4 Induces Systemic Sclerosis-like Effects in Subcutaneous Pump and in Subcutaneous Injection Models.



Panel D depicts skin histology from wild-type C57BL/6 mice treated with PBS control (top) or CXCL4 (bottom) for 28 days using daily subcutaneous injection model at 4x magnification (H&E). **Panel E** shows quantification of skin layers thickening after CXCL4 treatment for 28 days (n=5/group), as compared to the PBS controls (single asterisk denotes $P < 0.05$, double asterisks denote $P < 0.01$). The expression of pro-inflammatory marker CCL2 induced by CXCL4 after 28 day daily injection (n=5/group), compared to PBS, was measured using qPCR as depicted in **panel F**.

Supplementary Tables

Table S1. Disease Characteristics Patients with Systemic Lupus Erythematosus.	
N	109
Gender (female/male)	93/16
Age (years; mean ± SD)	45.8 ± 14.1
Disease duration (years; mean ± SD)	12.9 ± 10.4
Anti-dsDNA (Farr, E/ml; mean ± SD)	50.0 ± 145.9
Complement C3 (gr/l; mean ± SD)	0.97 ± 0.23
Complement C4 (gr/l; mean ± SD)	0.17 ± 0.08
ACR criteria[#] (N, (%))	
Malar rash	35 (32)
Discoid rash	37 (34)
Photosensitivity	49 (45)
Oral ulcers	8 (7)
Arthritis	71 (66)
Serositis	39 (36)
Renal disorder	40 (37)
Neurologic disorder	5 (5)
Hematologic disorder	75 (69)
Immunologic disorder	84 (78)
Antinuclear antibody	106 (98)

[#] American College of Rheumatology

Table S2. Disease Characteristics of Ankylosing Spondylitis Patients.	
N	93
Gender (% female)	73.4%
Age (years; mean ± SD)	44.9 ± 14.1
Disease duration (years; mean ± SD)	22.2 ± 17.3
HLA-B27 positive (%)	86%
Erythrocyte sedimentation rate (mm/hour)	29.7 ± 22.3
C-reactive protein	1.3 ± 1.2
BASDAI[#]	3.8 ± 2.5
TNFα neutralizing therapies (% usage)	28.1

[#] Bath Ankylosing Spondylitis activity index

Table S3. Mean CXCL4 Values in Cohorts.				
Cohorts	**Controls (n)**	**CXCL4 (pg/ml)**	**SSc Patients (n)**	**CXCL4 (pg/ml)**
Identification Cohort (plasmacytoid dendritic cells)	20	374.9 ± 45	53	66203 ± 3133
Identification Cohort (plasma)	22	90.4 ± 22	46	25143 ± 2533
Replication Cohorts (plasma)	129	92.6 ± 11	612[#]	23548 ± 2213
Early Disease (plasma)[‡]	128	101.4 ± 23	68	28781 ± 3211
Overall Mean	299	94.8 ± 18.7	779	25624 ± 2652

[#] 612 systemic sclerosis (SSc) patients are from University of Nijmegen, the Netherlands (n=148), Lund, Sweden (n=197), Milan, Italy (n=120), Verona, Italy (n=18), Ghent, Belgium (n=79), and Houston, TX (n=50).

[‡] Early disease patients are from Milan (independent to those in the replication cohort).

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2. Radstake TR, Blom AB, Sloetjes AW, et al. Increased FcgammaRII expression and aberrant tumour necrosis factor alpha production by mature dendritic cells from patients with active rheumatoid arthritis. Annals of the rheumatic diseases 2004;63(12):1556-63.
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